JOURNAL OF BACTERIOLOGY, Jan. 1992, p. 78–83
0021-9193/92/010078-06$02.00/0

Vol. 174, No. 1

Iron Uptake and Molecular Recognition in *Pseudomonas putida*: Receptor Mapping with Ferrichrome and Its Biomimetic Analogs

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Received 3 September 1991/Accepted 29 October 1991

The presence of an Fe^{3+}-ferrichrome uptake system in fluorescent *Pseudomonas* spp. was demonstrated, and its structural requirements were mapped in *Pseudomonas putida* with the help of biomimetic ferrichrome analogs. Growth tests, $^{55}Fe^{3+}$ uptake, and competition experiments demonstrated that the synthetic L-alanine derivative B5 inhibits the action of ferrichrome but does not facilitate Fe^{3+} transport, while the enantiomeric D-Ala derivative B6 fails to compete with ferrichrome. Contraction of the molecule's envelope by replacing L-Ala by glycine provided a synthetic carrier, B9, which fully simulates ferrichrome as a growth promoter. Sodium azide inhibited $^{55}Fe^{3+}$ uptake of the Gly derivative B9, suggesting an active transport process. These data demonstrate the chiral discrimination of the ferrichrome receptor and its sensitivity to subtle structural changes. They further confirm that receptor binding is a necessary but not sufficient condition for Fe^{3+} uptake to occur and suggest that binding to the receptor and transport proteins might rely on different recognition patterns.

Siderophores are small iron-binding molecules produced by most microorganisms grown in iron-deficient conditions (20). The siderophore-mediated iron uptake process involves (i) recognition of the ligand-metal complex by a receptor and (ii) its transport through one or two membranes to the site where the Fe^{3+} is removed and metabolized (20). Wild-type bacteria often possess several Fe^{3+} uptake systems, including receptors for iron-siderophore complexes which they do not synthesize (8). *Escherichia coli*, for example, possesses membrane receptors for ferrichrome, ferrioxamine B, and coprogen in addition to receptors for endogenously produced enterobactin and aerobactin (2). This strategy provides organisms with a competitive edge in the fight for sparsely available Fe^{3+} (8). Uptake of exogenous iron-siderophore complexes produced by soil fungi might play an important role in the rhizosphere ecology (4). This mechanism might be of importance for bacteria, such as fluorescent *Pseudomonas* spp., which have been shown to influence rhizosphere interactions (1, 26).

Ferrichrome is a hydroxamate Fe^{3+} chelator produced by many fungi (27). It consists of three glycyl and three δ-*N*-acetyl-δ-*N*-hydroxyl-L-ornithyl residues (22) and forms a complex of Λ-*cis* configuration when binding Fe^{3+} (18). Receptor recognition of this complex can a priori involve interactions with the domain around the metal center and/or with other regions of the molecular envelope. These features can be translated into simple molecular models which can be synthesized to simulate the characteristics essential for recognition and uptake (24). Biomimetic ferrichrome analogs that adopt the same absolute Λ-*cis* configuration around the Fe^{3+} center as ferrichrome were recently prepared, and it was shown that the L-alanine derivative B5 equals the natural siderophore as a growth promoter in *Arthrobacter flavescens* (25). In addition, the synthetic models helped to identify the domains essential for siderophore recognition. This article is the first in a series aimed at mapping the Fe^{3+} siderophore uptake machinery of fluorescent pseudomonads. The research described here aimed at (i) studying ferrichrome-mediated Fe^{3+} uptake in fluorescent pseudomonads and (ii) establishing the structural requirements for siderophore uptake with the help of biomimetic compounds as probes.

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MATERIALS AND METHODS

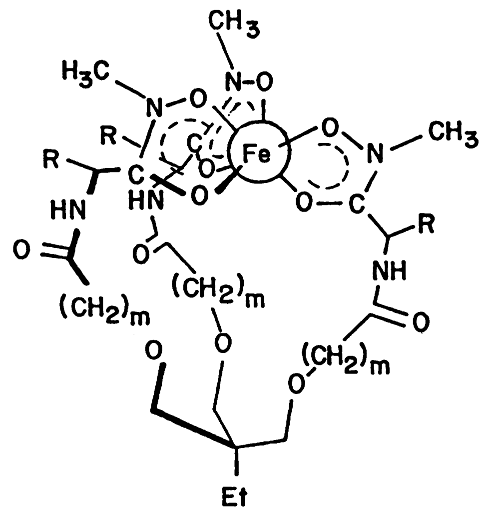
Bacterial strains. *Pseudomonas putida* WCS 358 and its siderophore-negative (Sid^-) Tn*5* mutant JM 218 were obtained from of P. Weisbeek, Utrecht, The Netherlands (11). *P. aeruginosa* $7NSK_2$ was obtained from M. Hofte, Ghent, Belgium (12). *P. putida* St3 was isolated in our laboratory (16).

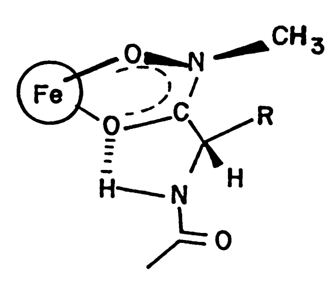
Siderophores. Ferrichrome was isolated by the method of Crowley et al. (7).

Synthesis. The ferrichrome analogs were prepared in three stages. This strategy involves (i) preparation of the C_3 symmetric anchor as an activated triscarboxylate ester; (ii) preparation of the single-stranded hydroxamate-bearing amino acid residues with free amino group terminals; and (iii) coupling of the anchor with the hydroxamate-bearing residues to form the final products. All compounds were purified by chromatography and fully characterized by their spectroscopic properties.

Plate tests. The antibiosis plate test was carried out as described by Buyer and Leong (3) with 0.6 ml of soft agar inoculated with approximately 10^3 CFU/ml poured over a modified King's B (MKB) medium (12) in 5-cm-diameter petri dishes. Twenty microliters of a 10^{-3} M solution of the tested desferri compound was dropped onto a 6-mm-diameter Whatman paper disk. The plates were incubated at 30°C and checked after 24 h.

For the iron-siderophore complex utilization test, the bacteria were grown in liquid MKB medium and 10^3 CFU/ml were plated on MKB plates containing 400 μM α,α′-dipy-





Biomimetic Ferrichrome Analogs

Code	m	R	amino acid component
B2	2	iBu	(L-leu)
B5	2	Me	(L-ala)
B6	2	Me	(D-ala)
B8	2	s.Bu	(L-ileu)
B9	2	H	(gly)
C5	1	Me	(L-ala)

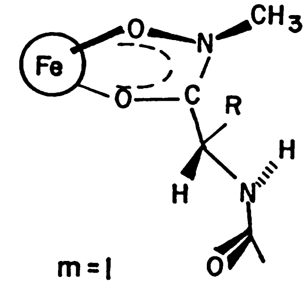


FIG. 1. Ferrichrome analogs.

ridyl or 2.7 mM EDDHA (ethylenediamine di-*o*-hydroxyphenyl acetic acid; Sigma, St. Louis, Mo.) treated as described by Rogers (21). The bacteria were also grown in liquid rhizosphere-simulating medium (RSM) and plated on RSM containing 201 μM EDDHA (5). Then, 10 μl of a 10^{-4} M solution of the various iron-siderophore complexes was dropped on a Whatman paper disk. The plates were incubated as described above.

Growth curves. The Sid$^-$ strain JM 218 was used to test the efficiency of bacterial growth promotion in an iron-free liquid medium. HEPES-succinate medium [HSM, containing (in grams per liter) succinate, 4; K_2HPO_4, 0.2; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.2; and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 11.91) was batch-treated for 4 h with Chelite N resin (10 g/liter; Serva, Heidelberg, Germany), decanted, and filtered through a 0.45-μm filter. The pH was then raised to 7.2 by adding 10 N NaOH, and the medium was autoclaved. Double-distilled water was used, and the glassware was washed with 6 N HCl, followed by thorough rinsing with double-distilled water.

Polypropylene test tubes (50 ml) were filled with 10 ml of the Chelite-treated HSM. The Fe^{3+} siderophores were added to a final concentration of 10^{-6} M, and the tubes were inoculated with about 4×10^7 CFU/ml and shaken in an inclined position at 28°C. Samples were taken at desired intervals, and turbidity at 620 nm was recorded. Each treatment was performed in triplicate.

^{55}Fe uptake. The procedure for determining ^{55}Fe uptake described by de Weger et al. (9) was used, with minor modifications. The mutant JM 218 was used in all experiments. The bacterium was grown in a half-strength standard succinate medium (SSM) to an A_{620} of 0.4, centrifuged for 15 min at 2,500 rpm, then resuspended in fresh half-strength SSM to a final A_{620} of 0.3, and incubated for 1 h in a water bath at 28°C. The labeled Fe^{3+} complex was added to a final concentration of 2 μM. Aliquots (0.6 ml) were taken in duplicate, layered onto a mixture of silicon oils, and centrifuged as described above. Counting was performed on a Beckman LS1801 counter.

RESULTS

Synthesis. The design of ferrichrome analogs was guided by two principles: biomimetic design and modular assembly. Biomimetics is based on the assumption that it is possible to reproduce the properties of natural products with simple, synthetic molecules by reproducing only the most essential structural features. Based on earlier studies on ferrichrome-mediated Fe^{3+} uptake (15, 28), we assumed that the domain around the bound Fe^{3+}, its envelope, and its absolute configuration, Λ-*cis*, are the essential features for recognition by membranous receptors, whereas the ring anchor is less important. Reproduction of these features with synthetic molecules was sought by adopting a modular "Lego" type strategy. This strategy allows systematic modifications of the compounds and their properties to be made by changing one component at a time. Following these principles, we synthesized ferrichrome analogs by replacing the nonsymmetric hexapeptide ring of the natural ferrichrome with a readily accessible C_3 symmetric triscarboxylate anchor, in which m = 1 or 2 (Fig. 1). Chirality and variety were

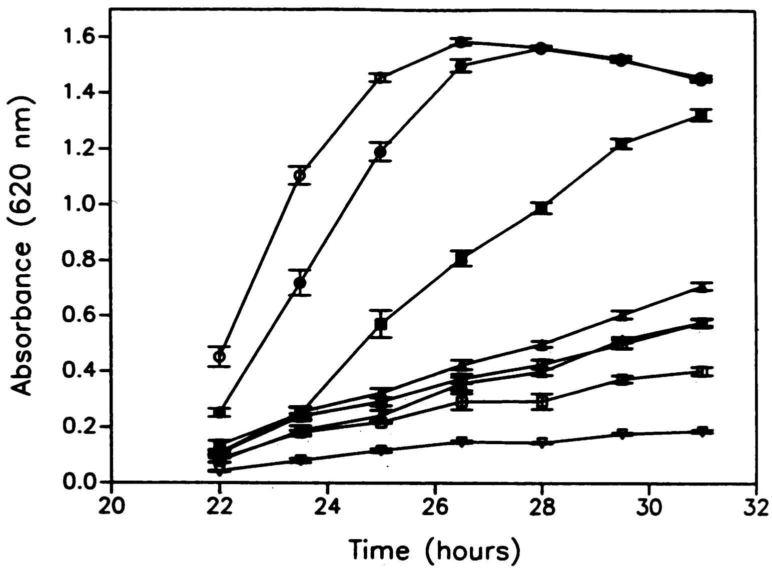


FIG. 2. Growth of mutant JM 218 in Chelite-treated HSM with ferrichrome (○) and the ferrichrome analogs B2 (⊞), B5 (□), B6 (■), B8 (△), B9 (●), and C5 (▲) as iron sources. Control (▽), no added iron source. Bars represent standard error.

introduced by extending these anchors with amino acid bridges, where R = H (Gly), CH_3 (L-Ala and D-Ala), $CH_2CH(CH_3)_2$ (L-Leu), or $CHCH_3CH_2CH_3$ (L-Ileu). Iron-binding hydroxamate groups were added by terminating the strands with *N*-methylhydroxamate groups.

These compounds were synthesized by a three-stage strategy. All compounds were fully characterized by their spectroscopic properties, such as infrared and nuclear magnetic resonance data. Titration experiments in conjunction with UV and visible-light spectroscopy established 1:1 binding stoichiometry to Fe^{3+} for all compounds. CD (circular dichroism) analysis of all L-amino acid derivatives showed preferential Λ-*cis* configuration, while derivatives composed of D-amino acid residues were found to preferentially assume the Δ-*cis* configuration. The chiral preference of the L-amino acid derivatives for the Λ configuration is estimated to be higher than 95% except for B5, for which it is about 80%.

Plate tests. Three strains of fluorescent pseudomonads (*P. putida* St3, *P. putida* WCS 358, and *P. aeruginosa* $7NSK_2$) and JM 218, the siderophore-deficient mutant derivative of *P. putida* WCS 358, were tested for their ability to utilize ferrichrome and the Fe^{3+} complexes of various synthetic analogs in solid media. Two types of tests were performed: (i) a plate test, in which all available iron except the added iron-siderophore complex is bound to a nonutilizable chelate (EDDHA or α,α′-dipyridyl); and (ii) a growth inhibition test, in which the desferri compounds were evaluated for bacteriostasis.

The siderophore utilization plate tests showed that the four strains were able to use ferrichrome. Growth was apparent around the paper disks only when they were impregnated with the siderophore. The RSM-EDDHA medium sustained higher and more rapid growth of the colonies than the MKB-EDDHA medium, with growth halos 25 to 35 mm and 10 to 15 mm in diameter after 24 h of incubation, respectively. This difference in zone size was probably due to differences in available Fe^{3+}-ferrichrome as a result of the higher concentration of EDDHA in the MKB medium. The Gly-based compound B9 also promoted growth of all strains, which developed halos of 15 to 20 mm in RSM-EDDHA. Very poor utilization of the chiral isomers B6 and B5 by all strains was seen in the RSM-EDDHA medium (halos of >10 mm). A series of compounds with chirality around the iron center but differing in the nature of the side chain and in the type of anchor (compounds B2, B8, and C5, Fig. 1) was also tested with the mutant strain JM 218 on MKB plates containing dipyridyl. Dipyridyl is an Fe^{2+} chelator that effectively immobilizes residual iron in the medium, thus preventing growth of Sid^- organisms. None of the analogs promoted growth except for the glycine-based compound B9, which

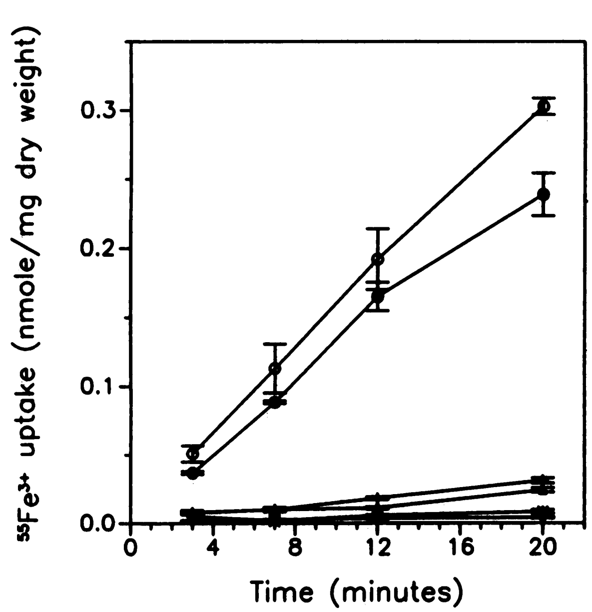


FIG. 3. $^{55}Fe^{3+}$ uptake mediated by ferrichrome (Fc, ○) and analog B9 (●) in the presence of 3 mM sodium azide (Fc, +; B9, ▲) and after growth in an iron-repleted medium (10 μM $FeSO_4$; Fc, *; B9, ▼). Bars represent standard error.

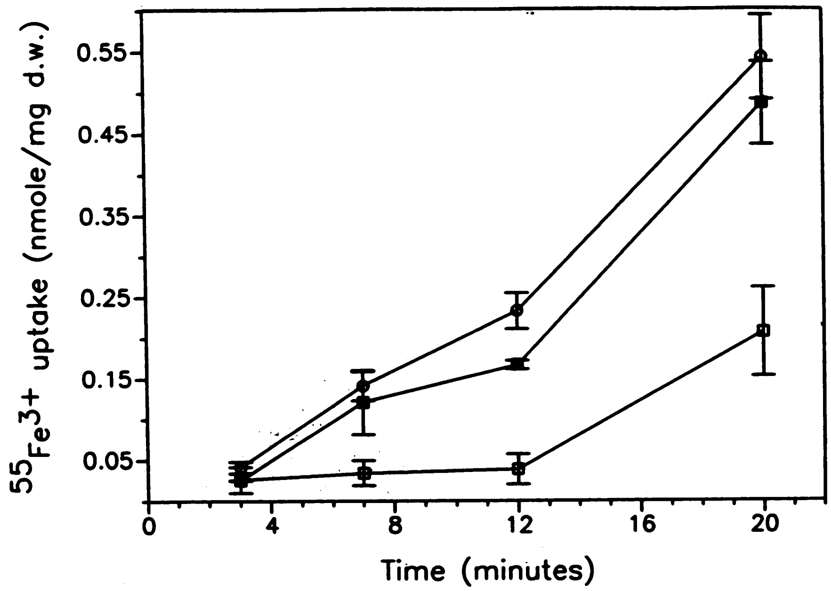


FIG. 4. Ferrichrome-mediated $^{55}Fe^{3+}$ uptake (2 μM, ○) and its inhibition with analogs B5 (20 μM, □) and B6 (20 μM, ■). Bars represent standard error.

sustained growth almost as efficiently as ferrichrome, as evidenced by growth halos of about 25 and 35 mm, respectively, after 24 h.

Desferriferrichrome was tested for inhibitory activity against strains St3, $7NSK_2$, WCS 358, and JM 218. The last strain was also used to evaluate growth inhibition by the synthetic analogs. Desferriferrichrome displayed no inhibitory effects. The synthetic siderophores, except for compounds B9 and B6, were all inhibitory to JM 218, with clear zones of growth inhibition being evident around the disks. This indicates that the iron present in the medium had been complexed by the added siderophore, becoming unavailable to the cell (3). Control plates of the Fe^{3+} siderophore analogs showed no growth inhibition. Compound B9 was not inhibitory to strain JM 218, whose growth was similar to that observed with ferrichrome. The chiral Δ-isomer B6 was not inhibitory either, but the colonies appearing on the petri dish developed more slowly and were therefore smaller at the end of the experiment.

Growth promotion in liquid medium. The growth-promoting effects of ferrichrome and of the synthetic analogs were also studied in liquid medium. Growth was tested in Chelite-treated HSM with strain JM 218. Since this strain does not excrete siderophores and does not react with CAS (chromeazurol S) reagent (23), no ligand exchange can occur between added and excreted siderophores. Growth of such Sid^- mutants is therefore proportional to the availability of the iron complex tested. As with the plate experiments, ferrichrome and compound B9 were very efficient growth promoters, while the controls showed very slow growth (Fig. 2). The growth curves obtained for ferrichrome and B9 were similar, and in both cases a generation time of 1.8 h could be calculated. The siderophores with a left-handed configuration (B5, B2, B8, and C5) about the iron center were much less effective, as evidenced by the much longer generation times (4.9 to 6.45 h). The enantiomer B6 was more effective than B5, with more rapid growth than with all other L-amino acid derivatives tested, but a doubling time of 2.75 h indicated that iron uptake was still the limiting factor.

^{55}Fe uptake. The mutant JM 218 was also used in short-term $^{55}Fe^{3+}$ uptake experiments. The bacterium efficiently promoted ferrichrome-mediated $^{55}Fe^{3+}$ incorporation. A total of 0.3 nmol/mg (dry weight [d.w.]) was incorporated after 20 min at a constant rate of 15 pmol/min/mg d.w. (Fig. 3). Iron uptake mediated by the analog B9 was slower than that mediated by ferrichrome, but it was much faster than that mediated by B6 (10 and 1 pmol/min/mg d.w., respectively), which also showed an almost constant rate of uptake. Iron uptake mediated by analog B5 was very low, showing saturation after 7 min of incubation. The final cellular iron concentration was about 1.5, 12, and 30 times lower for the analogs B9, B6, and B5, respectively, than that resulting from ferrichrome-mediated iron uptake. Addition of sodium azide to the medium inhibited iron uptake (Fig. 3), indicating this to be an energy-driven process. Ferrichrome- and B9-mediated iron uptake was also drastically reduced when the bacterium was grown in an iron-repleted medium (Fig. 3). From the data obtained in concentration-dependent uptake experiments, apparent K_m values of 0.05 μM for ferrichrome and 0.1 μM for B9 were calculated.

In order to establish whether the synthetic analogs made use of the same receptor as ferrichrome and to trace the structural requirements for ferrichrome-mediated iron uptake in *P. putida* JM 218, competition experiments involving ferrichrome-mediated $^{55}Fe^{3+}$ uptake were performed. Ferri-

TABLE 1. Inhibition of B9-mediated $^{55}Fe^{3+}$ uptake by ferrichrome and ferrichrome analogs[a]

Competitor	Concn (μM)	$^{55}Fe^{3+}$ uptake (nmol/mg d.w.)	% Inhibition by competitor
None	0	0.16	
Ferrichrome	2	0.019	88
B5	20	0.12	25
B6	20	0.123	23

[a] B9 used at 2 μM.

chrome-mediated uptake of $^{55}Fe^{3+}$ (2 μM) showed a 60% inhibition in the presence of cold Fe^{3+}-B5 at a concentration of 20 μM but was not inhibited by cold Fe^{3+}-B6 (Fig. 4). Compound C5, identical to B5 in its amino acid bridge but differing in its anchor, was also not inhibitory to ferrichrome-mediated $^{55}Fe^{3+}$ uptake. Analog B9 competed with ferrichrome, so that a concentration of 40 μM Fe^{3+}-B9 was needed to reduce the uptake of [$^{55}Fe^{3+}$]ferrichrome by 30%. In competition experiments between [$^{55}Fe^{3+}$]B9 and cold ferrichrome at equimolar concentrations, ferrichrome reduced the uptake of the synthetic siderophore by 88% (Table 1). Since the synthetic siderophore B9 is based on glycine, it is an achiral molecule that forms a racemic mixture of complexes. The effective concentration of the enantiomer adopting the Δ configuration was therefore only 20 μM. The siderophore analogs B5 and B6 inhibited uptake of the [$^{55}Fe^{3+}$]B9 complex, demonstrating that both enantiomers of the analog Fe^{3+}-B9 complex are taken up (Table 1).

DISCUSSION

Many bacteria, including fluorescent pseudomonads, can assimilate Fe complexes of siderophores which they do not produce (8). In this research, we have shown that bacteria belonging to the fluorescent pseudomonad group are able to utilize the ferrichrome siderophore as an Fe^{3+} source. The rate of ferrichrome-mediated iron uptake in strain JM 218 (Sid$^-$) was similar to that observed with exogenous pseudobactins and pyoverdines when tested with various strains of fluorescent pseudomonads (9, 13). Ferrichrome strongly promoted growth both on plates and in liquid medium. The high K_m values demonstrate the high affinity of the uptake system for ferrichrome, as they are in the range reported for ferrichrome (0.15 μM) and enterobactin (0.1 μM) in *E. coli* (10, 19). Whether the ferrichrome and pseudobactin uptake and transport systems in fluorescent pseudomonads have common components is currently under investigation. This possibility has to be considered, since the outer membrane receptor for pseudobactin WCS 358 in *P. putida* WCS 358 has homologous regions with the TonB-dependent proteins of *E. coli* and since various siderophore uptake systems may have common determinants (17). In any event, the finding that ferrichrome is an effective supplier of iron to fluorescent pseudomonads supports the hypothesis that scavenging of exogenous siderophores is a determining factor in the ecology of siderophores in the rhizosphere (4).

The series of biomimetic siderophores described here differ in the nature of their anchor ($m = 1$ versus $m = 2$ in Fig. 1), in their amino acid residues and their projecting side chains [H (Gly), CH_3 (Ala), $CH_2CH(CH_3)_2$ (Leu), or $CHCH_3CH_2CH_3$ (Ileu)], and in the chirality around the Fe^{3+} complex (L-Ala versus D-Ala). They allowed mapping of the structural requirements for recognition and transport. Using a Sid$^-$ mutant as the indicator organism, we found that among the chiral amino acid derivatives examined, only the L-Ala derivative B5 inhibited ferrichrome-mediated $^{55}Fe^{3+}$ uptake. This observation indicates competition between the L-Ala derivative and ferrichrome for the same receptor, although B5 failed to be subsequently transported. In an attempt to obtain a synthetic analog which would be transported into the cell, the lateral methyl group of B5 was reduced to hydrogen by replacing the L-Ala residue with glycine. The Gly derivative B9 facilitated $^{55}Fe^{3+}$ uptake into the cell. K_m values for Fe^{3+} uptake mediated by ferrichrome and B9 differed by only a factor of 2 (0.05 and 0.1 μM, respectively), while the difference in the competition experiments was much larger. Equimolar concentrations of ferrichrome inhibited [$^{55}Fe^{3+}$]B9 uptake by 88%, while 10-fold-higher concentrations of Fe^{3+}-B5 and Fe^{3+}-B6 inhibited [$^{55}Fe^{3+}$]B9 uptake by 25 and 23%, respectively. These data demonstrate that both configurations of Fe^{3+}-B9 are biologically active, but each makes use of a different receptor, although not necessarily of a different transport system.

The above findings illustrate the usefulness of biomimetic siderophores in differentiating between the recognition sites of membrane receptor and transport proteins. Differences in the structural requirements of receptors and transporters was first demonstrated by comparing ferrichromes with des(diserylglycyl) derivatives in *Neurospora crassa* (14). The performance of the synthetic compounds also demonstrates the sensitivity of the ferrichrome receptor to (i) the chiral sense of the siderophore (since the D-Ala derivative B6 failed to act as an inhibitor); (ii) the laterally projecting side chains (evidenced by the lack of activity of the L-Leu and L-Ileu derivatives B2 and B8 as inhibitors); and (iii) the details of the overall molecular shape (since the L-Ala derivative with the slightly modified anchor [$m = 1$] was inactive). The chiral discrimination recorded above is not surprising, since ferrichrome receptors in some bacteria and fungi have been shown previously to respond preferentially to ferrichromes with a Λ configuration over their Δ enantiomers (15, 24, 25, 28, 29). The discrimination between different lateral groups may readily be accounted for by differences in stereochemical requirements. However, the distinction between the L-Ala derivatives B5 and C5 is quite remarkable, as these two compounds differ mainly in the orientation of their amide linkages. While in B5 the amide groups are oriented radially (in the molecular cross section), with the amide NH pointing toward the Fe-O bond, in C5 the peptide groups are tangentially positioned (Fig. 2). A distinction between these two derivatives by the membrane receptor suggests either dipolar, attractive interactions between B5's projecting carbonyls and receptor functions or dipolar, repulsive interactions between the latter and C5 tangential amides. It was previously reported that B5 functions in *Arthrobacter flavescens* as an Fe^{3+} carrier equalling ferrichrome in biological activity (25). The differences in the biological activity of B5 in *A. flavescens* and in *P. putida* suggest that the ferrichrome uptake machinery of different organisms exhibit structural variation.

Recognition of the Fe^{3+}-ferrichrome complex in *P. putida* shows high chiral discrimination and sensitivity to subtle structural variations of the siderophore. Receptor binding is a necessary step, but it is not sufficient to ensure uptake, suggesting the necessity for further interactions with the receptor and/or the transport system.

The fact that the D-Ala derivative B6 failed to inhibit ferrichrome activity but acted as growth promoter strongly indicates the presence of an additional siderophore-mediated Fe^{3+} uptake system with preference for the Δ configuration. A possible candidate is the coprogen receptor. Experiments to examine this possibility are in progress.

ACKNOWLEDGMENTS

We thank Rahel Lazar for technical assistance.

This research was made possible by a grant from the U.S.-Israel Binational Agricultural Research and Development Fund (BARD). Abraham Shanzer is holder of the Siegfried and Irma Ullmann Professorial Chair.

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